EXTRACTION, PURIFICATION AND CONVERSION OF FLAVONOIDS FROM PLANT BIOMASS

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EXTRACTION, PURIFICATION AND CONVERSION OF FLAVONOIDS FROM PLANT BIOMASS

This invention relates to flavonoids and in particular to rutin enriched compositions prepared from plant biomass, which can be enzymatically converted to the more valuable flavonoids isoquercitrin and quercetin.

Background of the Invention

Plant flavonoids usually occur in plants as glycosides, although in some circumstances they may occur as free aglycones. Most glycosides are O-glycosides, with the most common monoglycoside being at the 7-position. Diglycosides usually have sugars at the -7 and -3 positions and occasionally the -7 and -4' positions. Other combinations and mono-O-glycosides exist but are less abundant. C-glycosides also occur in a more restricted distribution with C-6 and C-8 glycosides being the most common (Harbone, 1994).

Plant flavonoids have antioxidative properties (Bors *et al.,* 1990), cytostatic effects in tumorigenesis, and the ability to inhibit a broad spectrum of enzymes, such as angiotensin converting enzyme (ACE), protein kinase C, tyrosine protein kinase, and topoisomerase II. They are regarded as potential cancer preventatives and cardioprotective agents (Manach *et al.*, 1996; Skibola and Smith, 2000). Their potential use as anti-inflammatory or antiviral agents has also been examined (Middleton and Kandaswami, 1993). Backhaus (1995a) claimed that bioflavonoids, especially rutin, citrin, quercetin, hesperidin or derivatives were responsible for the inactivation of protein-cleaving enzymes (such as hyaluronidase and/or collagenase), which promote skin-aging processes. These compounds may be used for general skin care or cosmetic surgery. It is reported that rutin, quercetin, isoquercitrin, catechin and other compounds also prevent and ameliorate the aging phenomena of the skin (Arata, 1992). Midori (1994) claimed that, together, quercetin glycoside, divalent

Although most of the details of the industrial production of rutin are proprietary and not described in the open literature, we are aware that Merck GmbH extracts rutin from fava d'anta for commercial purposes. Heywang and Basedow (1992) of Merck GmbH Germany, extracted rutin from shoots of fava d'anta (*Dimorphandra*) with 1,4-dioxane under reflux. Rutin was recovered by crystallization at room temperature. Dioxane is, however, considered carcinogenic.

Huo (Chinese Patent 1217329, 1999) described an extraction of rutin from tartary buckwheat seeds by washing with water, coarse grinding, coarse screening, soaking in water, drying in the air, fine grinding, soaking in edible alcohol, extracting below 60°C, and filtering. Balandina *et al.* (1982) extracted rutin from buckwheat seeds with hot water to remove the desired product and crystallized it.

Zhai (Chinese Patent CN 1160048, 1997) described the extraction of rutin from *Flos sophorae* by soaking with saturated limewater containing 1 - 10% borax, and precipitating at pH 1- 6 by adding HCl.

Matsumoto and Hamamoto (1990) recovered rutin from *Sophora augustifolia* buds with methanolic extraction, adsorption onto activated carbon followed by desorption, by elution with 1% ammonia in 40% ethanol, and recrystallization from 20% ethanol.

Liu (1991) described a method of extracting rutin from Japanese Pagoda tree (*Sophora japonica*) buds by pulverizing, streaming in limewater, neutralizing the supernatant, cooling, filtering, washing, and drying the precipitates. The yield was 14.2% (wt/wt) and the product contained 95.1% (wt/wt) rutin.

Sloley *et al.* (2000) reported that, while hypericin is regarded as a marker chemical for extracts of leaves and flowers of *Hypericum perforatum* (St. John's wort), other compounds such as hyperforin, hyperoside, rutin and quercetin are presented in much higher concentrations. They also found that chemical composition profiles varied greatly among different extracts. However, free-radical-scavenging capacity

similar in activity to the epipodophylotoxins widely used in cancer therapy (Skibula and Smith, 2000).

Ishige *et al.* (2001) showed that many flavonoids and related polyphenolic compounds protected the mouse hippocampal cell line HT-22 and rat primary neurons from oxidative stress caused by glutamate. This finding is significant because nerve cell death from oxidative stress has been implicated in a variety of pathologies, including stroke, arteriosclerosis, trauma, and Alzheimer's and Parkinson's diseases. Their data show that some flavonoids (quercetin, kaempferol, and fisetin) are quite protective, while others (rutin, chrysin, and apigenin) are inactive. Quercetin alters glutathione (GSH) metabolism and inhibits reactive oxygen species (ROS) in a cell culture model of oxidative stress. Its mechanism of action is similar to that of propyl gallate and methyl caffeate, but different from that of vitamin E. Noroozi *et al.* (1998) reported that quercetin is more potent than rutin and vitamin C in countering against oxidative DNA damage.

Ashida *et al.* (2000) reported that dietary flavonols (quercetin and rutin) and flavones suppress antagonistically the transformation of aryl hydrocarbon receptor (AhR) induced by dioxin. Quercetin is more potent that rutin in counteracting the toxicity of this environmental contaminant. In the area of anticarcinogenicity, phase I enzymes oxidize, reduce or hydrolyze carcinogens, and phase II enzymes conjugate or otherwise affect carcinogens. Valerio *et al.* (2001) demonstrated that quercetin is a phase II enzyme inducer that stimulates phase II detoxifying activities. Phase II enzymes can also scavenge strong oxidants, and scientific interest has been directed toward their activity as a means of decreasing the risk of cancer. Use of phase II enzyme inducers, many of which are found in common foods, is one way to increase phase II enzyme activities in body tissues.

Agullo *et al.* (1997) reported that quercetin was an effective inhibitor of phosphatidyl inositol 3-kinase (PI 3-kinase; an enzyme involved in cell multiplication and transformation). Luteolin, apigenin and myricetin also exhibit such activity. Inhibition of PI 3-kinase may be linked to the antitumor properties of these

Naturally occurring isoquercitrin (quercetin-3-O-beta glycoside) can be extracted from flowers of levant cotton (*Gossypium herbaceum*), *Waldsteinia fragarioides* (Michx) Tratt (*Rosaceae*), *Spartium junceum* L. (*Fabaceae*) (Yesilada *et al.*, 2000), and horse chestnut (*Aesculus hippocastanum*). It is also found in celery seed, fennel seeds, horsetail, red clover and St. John's wort. Isoquercitrin has shown to possess several biological activities, including inhibition of angiotensin converting enzyme (ACE), inhibition of prostaglandin synthesis, and antiviral activity (Abou-Karam and Shier, 1992).

The role of bacterial enzymes in the digestive absorption of flavonoids is important because mammalian tissues are unable to synthesize such hydrolases. Griffiths and Barrow (1972) have shown that flavonoid glycosides ingested by germ-free rats were recovered unhydrolyzed in the feces. Hydrolysis of the sugar-aglycone bond takes place in the distal ileum and the caecum.

During absorption across the intestinal membrane, flavonoids are absorbed in the aglycone and/or glucoside forms and are partly transformed into their glucuronides, sulfates or methoxylates (Manach *et al.*, 1998). Free quercetin could not detected in blood plasma. The small fraction of flavonoids that is absorbed is metabolized by liver enzymes resulting in polar conjugates being excreted in the urine or returned to the duodenum via the gallbladder. The largest fraction of ingested flavonoids, that is not absorbed, is degraded by the intestinal microflora. The bacterial enzymes catalyze several reactions, including hydrolysis, cleavage of the heterocyclic oxygen-containing ring, dehydroxylation, and decarboxylation. Several phenolic acids are produced, depending on the structure of the flavonoid involved. Phenolic acids can then be absorbed and subjected to conjugation and O-methylation in the liver and may then enter into the circulation (Manach *et al.*, 1996).

Crespy *et al.* (1999) demonstrated that quercetin and isoquercitrin are much more bioavailable than rutin. Rutin is absorbed more slowly than quercetin, isoquercitrin and isorhamnetin because it must be hydrolyzed by the caecal microflora, whereas

no previously disclosed method for the processing of buckwheat foliar material for recovery of flavonoids and for the further biotransformation of such flavonoids to highly bioavailable, performance-enhanced, high-value products such as isoquercitrin and quercetin. Previously disclosed are only classical laboratory procedure for the extraction and purification of rutin.

Usually, the concentrations of naturally occurring isoquercitrin and quercetin found in biological systems are much lower than that of rutin. Isoquercitrin and quercetin extracted from biological systems demand much higher prices due to their rareness and bioavailability. There is not presently any commercially feasible technology for the biotransformation of rutin (regardless of the source) to highly bioavailable, performance enhanced and high value products such as isoquercitrin and quercetin.

Summary of the Invention

It is an object of this invention to provide an isoquercitrin-enriched composition derived from rutin, and to provide such a composition economically in commercial amounts sufficient to permit their use in functional foods, nutraceutical, natural health products, cosmetics and pharmaceutical applications.

It is a further object of this invention to provide a composition derived from rutin that is enriched in controlled proportions of isoquercitrin and quercetin, and to provide such a composition in commercial amounts sufficient to permit their use in functional foods, nutraceutical, natural health products, cosmetics and pharmaceutical applications.

It is a further object of this invention to provide a method whereby the yield of isoquercitrin can be maximized by inhibiting the conversion of isoquercitrin to quercetin. In the invention this is accomplished by the addition of an inhibitor of the ß-D-glucosidase activity present in naringinase preparations.

It is a further object of this invention to provide a process for deriving rutin from buckwheat, and in particular to provide such a process deriving rutin from the

suitable for enzyme incubation during an incubation period; terminating the incubation period by changing the conditions of the solution to conditions unsuitable for enzyme activity. These changes include lowering the pH and increasing the temperature of the solutione. Adjusting the duration of the incubation period controls the proportion of isoquercitrin in the composition.

In a third aspect the invention provides a composition enriched in isoquercitrin prepared by a process comprising providing a solution having rutin suspended therein at conditions suitable for enzyme incubation; adding an enzyme preparation comprising the enzymes naringinase or α-L-rhamnosidase to the solution; maintaining the conditions of the solution suitable for enzyme incubation during an incubation period; terminating the incubation period by changing the conditions of the solution to conditions unsuitable for enzyme incubation. For optimal yields, the temperature should be in the range of 50 – 55°C and should not exceed 65°C. Adjusting the duration of the incubation period controls the proportion of isoquercitrin in the composition. The incubation period is optimally in the range of 1 – 48 hrs. Lowering pH and increasing the temperature of the solution terminates the incubation period by denaturing the enzyme preparation.

The proportion of isoquercitrin in the composition can be up to about 95%. The enzyme incubation with the enzyme preparation containing α-L-rhamnosidase and β-D-glucosidase also converts rutin to quercetin. The incubation period can be adjusted to provide a composition enriched with both isoquercitrin and quercetin in varying proportions.

Conveniently and economically the enzyme preparation can be naringinase, which is commercially available and economical. Naringinase is sold with a guaranteed content of the enzyme β-D-glucosidase for various commercial uses. Contrary to the prior art revealed by Narikawa *et al* (1998), it was found that naringinase from *Penicillium decumbens* was able to cleave sugar from the rutin.

tyrosine protein kinase inhibitory, topoisomerase II inhibitory and protein-cleaving enzyme inhibitory properties.

The bioactive properties of the isoquercitrin-enriched product produce by the process of the present invention will be useful as an additive in health foods, pharmaceuticals products, nutraceuticals and cosmetics. When added to products, the bioactive properties will be useful in the prevention and treatment of diseases and health problems, including, but not limited to cardiovascular disease, stroke, capillary fragility, arteriosclerosis, trauma, oxidative stress, hypertension, elevated cholesterol, elevated triglycerides, hyperglycemia, types II diabetes, obesity and related disorders, Alzheimer's disease, Parkinsonism, asthma and some cancers.

The present invention also offers processing and product flexibility enabling economical manufacture and satisfaction of market preferences.

These and other objects, features, and advantages of the invention become further apparent in the following detailed description of the invention that illustrates, by way of example, the principles of this invention.

Brief Description of the Drawings

While the invention is claimed in the concluding portions hereof, preferred embodiments are provided in the accompanying detailed description which may be best understood in conjunction with the accompanying diagrams where like parts in each of the several diagrams are labeled with like numbers, and where:

Fig. 1A illustrates the chemical structural formula for rutin;

Fig. 1B illustrates the chemical structural formula for isoquercitrin;

Fig. 1C illustrates the chemical structural formula for quercetin;

Detailed Description of the Illustrated Embodiments

The present invention provides a method for the production of high value bioavailable flavonoids from plant biomass. As described above, flavonoids have been shown to have a range of useful bioactive properties. One of the problems in the use of flavonoids in therapeutic applications is that they normally exist at low concentrations in nature. In order to use flavonoids as additives in pharmaceutical, nutraceutical or other health products, a method for purifying flavonoids is required.

In the present invention, the flavonoid rutin is recovered by standard biochemical methods. Rutin is then converted to isoquercitrin and quercetin through the action of the enzyme preparation naringinase. A further refinement of the present invention shows that the yield of the intermediate product isoquercitrin can be enhanced by selectively inhibiting the ß-D-glucosidase activity present in the naringinase preparation, using the food additive d-Δ-gluconolactone.

The following examples and figures illustrate the operation of certain embodiments of the present invention so that it may be more readily understood.

With specific reference now to the figures in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice. It is stressed that the particulars shown are by way of example and for the purposes of illustrative discussion,

precipitation have not earlier been described as a finished product. The value of a rutin-enriched composition has not earlier been recognized.

Example 1:

Aqueous Extraction, Concentration and Precipitation of Rutin from Buckwheat Leaf Material

Following harvest and drying, buckwheat leaves were prepared for extraction by grinding on a Wiley mill to pass a 2mm screen. One kg of ground buckwheat leaves (rutin content is 3.74%, dry weight basis) were extracted in 10 L of water with continuous stirring at 90°C for 1 hour. The resulting suspension was filtered, and the filtercake was washed 2 times with 300 ml of hot (95°C) water. The wash filtrate was combined with the extract to give a combined extract volume of 8.6L. The aqueous extraction procedure recovered 36% of the available rutin from the leaves. The extract was concentrated under reduced pressure to approximately 1/5 or 1/10 of the original volume. The concentrated extract was stored in the refrigerator (4°C) overnight at which point the flavonoids precipitated out of solution. The precipitated material was collected following centrifugation at 7,000 x g and filtration of the supernatant. The pellet was subsequently freeze-dried. The rutin content of the precipitate was determined by dissolving an aliquot of the dried product in methanol and analyzing by RP-HPLC. From the HPLC results, we have concluded that 60% of the available rutin in the concentrated aqueous extract (reduced to 1/5 and 1/10 of original volume) can be recovered in the precipitate (pellet).

Example 2:

Aqueous Alcohol Extraction, Concentration and Precipitation of Rutin from Buckwheat Leaf Material

Following harvest and drying, buckwheat leaves were prepared for extraction by grinding on a Wiley mill to pass a 2mm screen. One kg of ground buckwheat leaves

of the product. Rutin content can be increased to about 70 % or higher after repeat solubilization/crystallization without using chromatography.

Example 4:

Optimization of Rutin Extraction from Buckwheat Leaf Material

Buckwheat leaves obtained as noted in Example 2 were extracted with the following solvents in a Solid:Solvent ratio of 1:20 for 4 hours at 60°C: Water, 30%(v/v) methanol/ 70% (v/v) water, 50%(v/v) methanol, 70%(v/v) methanol/ 30% (v/v) water, 85%(v/v) methanol/ 15%(v/v) water, and 100% methanol. The resulting extracts were then filtered and analyzed by RP-HPLC. The methanol content in the extraction solvent had a significant effect on the extraction efficiency of rutin from buckwheat leaves (Table 1.)

The optimal extraction conditions for the recovery of rutin from buckwheat leaves were determined from a series of optimization studies. The effects of varying the alcohol content of the extracting solvent, as well as the extraction temperature, extraction time and the solid to solvent ratio were significant. Tables 1-3 summarize some of these results.

Table1: Effect of the concentration of methanol in the extraction solvent on rutin extraction efficiency using 1:20 solid:solvent ratio, and a 4 hour extraction at 60°C.

% methanol in solvent (%, v/v)	Extraction Efficiency of Rutin (%)
0	1.0
30	29.2
50	86.5
70	94.1

Table 3: Effect of extraction time and solid:solvent ratio on rutin extraction efficiency using 70%(v/v) Methanol extraction solvent at 50°C.

Solid:Solvent Ratio	Extraction Time (hrs.)	Extraction Efficiency of Rutin (%)
1:10	2	86.4
1:20	2	91.8
1:30	2	94.5
1:10	3	92.7
1:20	3	92.7
1:30	3	95.4
1:10	4	90.0
1:20	4	99.9
1:30	4	96.3

[Extraction Efficiency of Rutin (%)
= (total rutin in extract / total rutin in the starting material) X 100]

Conversion of Rutin to Isoquercitrin and Quercetin

Fig. 1A illustrates the molecular make-up of rutin. Reaction of the enzyme α-L-rhamnosidase causes a biotransformation from rutin to the isoquercitrin of Fig. 1B by removing the first sugar on the bottom right hand side. To illustrate, the enzyme α-L-rhamnosidase essentially makes a conceptual incision along line A – A' in Fig. 1A.

Reaction of the enzyme β-D-glucosidase causes a biotransformation from the isoquercitrin of Fig. 1B to the quercetin of Fig. 1C by removing the sugar on the

composition is trace:1:3.38, and the composition comprises well over three times as much quercetin as isoquercitrin.

It can be readily seen that by adjusting the incubation period the proportions of rutin, isoquercitrin, and quercetin can be adjusted. The incubation times are measured in hours, such that considerable time latitude is available, allowing for conversion on a large scale in commercially significant quantities.

As shown in Example 6, after one day of enzymatic transformation, commercially sourced rutin (purity of 95% by weight) was converted to an isoquercitrin-enriched composition having weight ratios of rutin/isoquercitrin/quercetin of 0.1:1.0:0.2.

As shown in Example 7, commercial rutin was converted from the high rutin composition of Fig. 3A to the high isoquercitrin and quercetin composition of Fig. 3B.

As shown in Example 8, the high isoquercitrin and quercetin composition produced in Example 7 was further purified by Deltaprep C-18 chromatography, and high purity (95% +) isoquercitrin was obtained with a yield of 75% of the isoquercitrin in the starting material.

As shown in example 10, the ß-glucosidase in the naringinase can be inhibited by the addition of D-Δ-gluconolactone, or other food facilitator, without affecting the activity of alpha-rhamnosidase. D-Δ-gluconolactone has been used for years as a food additive, for example as a coagulant in the production of tofu. In the present invention D-Δ-gluconolactone adds flexibility and further assurance that the process will produce high isoquercitrin yield. Selective inhibition of ß-glucosidase, or selective separation of alpha-rhamnosidase from the naringinase for the production of isoquercitrin is within the scope of the claimed invention.

As shown in example 11, a medium-scale process is able to produce a highly enriched isoquercitrin product from buckwheat leaves. Thus, novel products can be produced from low value plant biomass.

Example 5:

Conversion of Rutin to Isoquercitrin and Quercetin using Enzymatic Hydrolysis

By manipulating the biotransformation conditions, we were able to convert the flavonoid-enriched intermediates to products containing different profiles of rutin/isoquercitrin/quercetin.

The freeze-dried rutin product (approximately 60% Rutin) produced in Example 2 was used for the enzymatic conversion experiments. A quantity of 5 grams of dry rutin product was dispersed in 500 ml of water (solid:liquid ratio = 1:100). The dispersion was heated to 80°C and the pH adjusted to 4. The dispersion was then equilibrated at 50°C, followed by addition of food-grade naringinase enzyme powder (Amano Pharmaceutical Co., Ltd; Japan).

The naringinase preparation contains 150 units of beta-glucosidase or naringinase activity as described in the specifications from the supplier. A dosage of 66 mg of Amano naringinase was used per g of rutin in this trial. The enzymatic incubation was maintained at 50°C with continuous stirring, for the appropriate length of time. Once the incubation time was complete, the enzyme was inactivated by adjusting the pH of the solution to 2.5 and then heating to 80°C for 10 minutes with continuous stirring. After 10 minutes at 80°C, the solution was cooled to room temperature, and the pH adjusted to 7. The enzyme-converted product was then dried by spray drying, freeze drying or other appropriate means.

Table 4 summarizes the experimental conditions required to prepare products containing various rutin/isoquercitrin/quercetin profiles. The starting material described here was previously freeze-dried for convenience reason. The precipitate (pellet) recovered prior to the drying step in the Example 2 is also suitable as a starting material for the Example 5. The enzymatic conversion can be applied at different stages, i.e., prior to the extraction of flavonoids, after aqueous extraction, after pre-concentration, or after precipitation. Flavonoid profiles

temperature, and the pH adjusted to 7. A 1.0 ml aliquot of the extract was removed for RP-HPLC analysis of the composition of the final product. The remaining extract was freeze-dried. The HPLC results indicated that the pure rutin standard had been converted to a product containing a rutin/isoquercitrin/quercetin profile of 0.12:1:0.21 (weight ratio).

Example 7:

Scale-up Conversion

Commercial rutin purchased from Street Chemicals was used for the enzymatic conversion similar to that described in Example 6. Concentrations of rutin and isoquercitrin in the commercial rutin are shown in Fig. 3A. A quantity of 109g of rutin was dispersed in 4000 ml water. The dispersion was heated to 80°C and the pH adjusted to 4. The dispersion was then equilibrated at 55°C, followed by addition of 24.2g of naringinase enzyme powder. The enzymatic incubation was maintained at 55°C with continuous stirring, for 24 hours. Once the incubation time was complete, the enzyme was inactivated by adjusting the pH of the solution to 2.5 and heating to 80°C for 10 minutes with continuous stirring. After 10 minutes at 80°C, the solution was cooled to room temperature, and the pH adjusted to 7. The solution was stored in the refrigerator (4°C) overnight. The solids recovered from centrifugation were freeze-dried. A quantity of 61.8 g of dry matter was obtained. The chromatogram of this product is shown in the Fig 3B.

to isoquercitrin. The HPLC results of the initial St. John's Wort extract indicated a rutin/isoquercitrin/quercetin profile of 0.47:1:0.21 (weight ratio). The enzyme converted product was found to contain a rutin/isoquercitrin/quercetin profile of trace:1:0.18 (weight ratio) which indicated that all rutin present in the initial extract had been converted to isoquercitrin and quercetin.

Example 10: Large-scale conversion of rutin to isoquercitrin using naringinase and D-delta- gluconolactone

Pharmaceutical grade rutin (38.15 g) from ICN was dispersed in 3.5 L of deionized water. Naringinase (8.47 g in 100 ml water) and D-Δ-gluconolactone (6.23 g in 100 ml water) solution were prepared. D- Δ-gluconolactone solution as added to the rutin:water mixture. The pH of the mixture was 4.0. The mixture was then heated to 80°C and incubated 2 hr. The temperature was then reduced to 55°C and the naringinase solution added. The mixture was incubated for 24 hr at 55°C with stirring. To stop the reaction, the pH was decreased to 2.5 and the mixture heated to 80°C for 10 min. The mixture was allowed to cool to room temperature and then the pH was adjusted to 7.0. The mixture was then refrigerated overnight to induce formation of a precipitate and the precipitate was allowed to settle. The precipitate was collected by centrifugation and then freeze-dried (the PPT1 fraction). The supernatant fluid was concentrated and then re-centrifuged. The resultant pellet was also freeze-dried (the PPT2 fraction). Three batches were prepared in this manner. The rutin, isoquercitrin and quercetin in different fractions from each batch were analyzed by HPLC. The data are presented in Table 5.

The data in table 5 demonstrates that rutin and quercetin appear as minor components, whereas isoquercitrin is the principal product observed after enzymatic conversion. For example, a total of 77.72 g of isoquercitrin and 0.53 of quercetin were produced from the three batches that were processed by the method of example 10. The majority (61.8 g) of isoquercitrin appeared in the PPT 1 fraction. The conversion process was very efficient, as only 0.2009 g of rutin was left unconverted by the

approximately 4 L of hot 70% methanol. The filtrates were combined and the volume reduced using a rotary evaporator until the volume was 1/5 of the original volume. The concentrated extract was refrigerated and allowed to precipitate overnight. The mixture was then stirred, and then centrifuged to collect the rutin.

Based on previous analysis, rutin content was estimated to be 33.66 g from 1 kg of starting leaf material. The amounts of enzyme and inhibitor used were based on these estimates, and were similar to previous conversions (7.36 g naringinase; 6.23 g D-Δ-gluconolactone; 3.5 L water).

The rutin precipitate was added to 3.5 L of water, and D-Δ-gluconolactone solution added. The pH of the mixture was 4.0. The mixture was then heated to 80°C and incubated for 2 hr. The mixture was then cooled to 55°C and the naringinase solution added. The mixture was then incubated at 55°C for 24 hrs. The reaction was stopped by reducing the pH to 2.5, and then incubating at 80°C for 10 min. The mixture was cooled to room temperature and the pH adjusted to 7.0. The mixture was then placed at 4°C overnight to allow a precipitate to form. The precipitate was collected by centrifugation as in Example 10.

The precipitate pellet was dissolved in methanol at 55°C with stirring. The solution was filtered to remove insoluble material. The filtrate was then concentrated as much as possible without allowing the mixture to bubble in the concentration vessel. At this point, 1.5 L of hot water was added to the mixture, and the material re-precipitated by incubation at 4°C for 2 days, and the precipitate collected by centrifugation. The re-precipitated material was then washed with hot water and precipitated for a third time. This final precipitate was freeze-dried to form a final product.

Note Respecting Methods and Examples

The buckwheat flavonoid content was determined by reverse phase high performance liquid chromatography (RP-HPLC) on a Waters Symmetry C-18 column